Studies on the effect of beta sitosterol on insulin signaling molecules and glucose oxidation in liver of high fat diet and sucrose-induced type-2 diabetic adult male rats

SYNOPSIS OF THESIS

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BACKGROUND

Diabetes mellitus is not a single disease, but a group of metabolic disorders affecting a huge number of populations in the world and is mainly characterized by chronic hyperglycemia, resulting from defects in insulin secretion (Type I) or its action (Type II). It is predicated that the number of diabetic persons in the world could reach up to 366 million by the year 2030 (Patel *et al*.,2012) The prolonged exposure to chronic hyperglycemia in diabetes leads to the development of complications of cardiovascular, renal, neurological, and visual systems to produce diseases such as cataract, retinopathy, neuropathy, and nephropathy (Patel *et al*., 2012) The frequency of this disorder is on the rise globally, is likely to hit 300 million by 2025 with India projected to have the largest number of diabetic cases (Gupta and Phatak, 2003) Although several oral antidiabetic therapies such as biguanides (phenoformin and metformin), sulfonylureas (glibenclamide and glipizide), and thiazolidinediones are widely used along with insulin for the treatment of diabetes mellitus, there are certain limitations due to high cost and side effects such as development of hypoglycemia, weight gain, gastrointestinal disturbances, liver toxicity, and impaired renal function (Raheja, 1977; Sharma *et al*., 2011)

Liver is the largest organ of the body that carries out all important metabolic processes of carbohydrate, protein, and lipid. It plays an important role in the regulation of glucose hemostasis by storing glucose in the form of glycogen during hyperglycemic status and converting stored glycogen and amino acids to glucose then release into bloodstream during hypoglycemic status.

The liver is an insulin-controlled organ: Its behavior changes depending on the level of the hormone insulin in the body and how sensitive the liver is to that insulin. After eating, blood glucose levels rise, which in people without diabetes triggers the pancreas to release insulin into the blood. Insulin gives the signal for the body to absorb glucose from the blood. Most cells just use the glucose to supply them with energy. It also

contains many antioxidant enzymes that protect cells from being lysed and also reduces oxidative stress under diabetic condition.

Recently plant-based therapies have been focused for the treatment of DM because of low cost, less toxicity with fewer side effects and there is a lot of scope for alternative therapy, considering the side effects associated with conventional drugs (Satyanarayana *et al.*, 2001)

The recommendations of the World Health Organization on the use of alternative medicines for treating diabetes provide an impetus for research in this area (WHO, 1999) The center of attention of research in diabetes includes discovering newer antidiabetic agents and isolating the active compounds from herbal sources that have been recognized to have antidiabetic properties as have been described in ancient texts. Herbs have accelerated the global efforts to harness and harvest medicinal plants having multiple beneficial effects. Some pre-clinical and clinical studies have confirmed their hypoglycemic effect and actions like repair of β-cells of islets of Langerhans (Saxena and Vikram, 2004)

Phytosterols are a group of plant sterols including β-sitosterol, stigmasterol, campesterol, and brassicasterol with β-sitosterol being a major isomer. They are sold as a functional cholesterol-lowering nutraceutical in Europe, the United States, and Australia. One of the major applications is the addition of phytosterols into spreads and vegetable oils. (Hovenkamp *et al.*, 2008). Plant sterols are present in all human dietary vegetable oils, seeds, fruits, nuts, cereals and legumes. Among the various phytosterols, interest in the biological activity of b-sitosterol (24-ethyl-5- cholestene-3-ol), the most common sterol, has increased in recent years.

Beta-sitosterol is a naturally occurring plant sterol, ubiquitously found in many plants (Pegel, 1997) It has been reported to elicit a multitude of bioactivities, which include, anti-inflammatory (Backhouse *et al.*, 2008) anticancer (Awad *et al.*,2007) antioxidant (Yoshida and Niki, 2003) angiogenic (Choi *et al.*, 2002; Moon *et al.*,1999) chemopreventive (Imanaka, 2008) and immunomodulatory activities (Bouic *et*

al.,1996, 1999, 2001 and Wilt, 1999) Beta-sitosterol is indicated for benign prostate hyperplasia (Weizel and Richter, 1997) coronary heart disease (Malini and Vanithakumari, 1990) and hypercholesterolemia (Ivorra *et al*., 1988). Rats supplemented with oral beta-sitosterol showed an increase in fasting insulin level, decreased fasting glucose level, improved oral glucose tolerance, and increased insulin release from isolated rat pancreatic islet cells (Ivorra *et al*., 1990; Sutherland *et al*.,199). It was reported that plasma plant sterol concentrations were significantly lower in human diabetics compared to normal controls and serum insulin levels were inversely correlated with plasma plant sterol concentration in diabetics (Ivorra *et al*.,1988) Though the studies have shown the antidiabetic properties of β-sitosterol using experimental and invitro model, the molecular mechanisms underlying the specific effects of β-sitosterol on insulin signaling molecules and glucose oxidation against high fat diet and sucrose-induced insulin resistance has not been explored.

Hence, the present study was designed to assess the possible role of β-sitosterol on insulin signal transduction and glucose oxidation in high fat diet and sucrose-induced type-2 diabetic adult male rats.

HYPOTHESIS

β-sitosterol may exhibit antidiabetic property through restoration of insulin signal transduction, glucose uptake and oxidation in the liver, as well as serum lipid profile in type-2 diabetic adult male rat.

OBJECTIVES

To identify the effects of β-sitosterol on

- Glucose and insulin tolerances, serum lipid profile, liver & kidney function markers in type-2 diabetic adult male rat.

- Carbohydrate metabolic enzymes (glucokinase, glucose -6- phosphatase, phospho enol pyruvate carboxy kinase, glycogen synthase and glycogen phospharylase)
- Insulin signalling molecules, GLUT2 translocation, glucose uptake and oxidation in the liver of type-2 diabetic adult male rat.

MATERIALS AND METHODS

Animals

Animals were maintained as per the National Guidelines and Protocols approved by the Institutional Animal Ethics committee (IAEC no: 011/2016, dated 04.07.2016). Healthy adult male Wistar albino rats of Wistar strain (*Rattus norvegicus*) (150–180 days old weighing 180–200 g) were used in this study and maintained in clean polypropylene cages at the Central Animal House Facility, Meenakshi Medical College and Research Institute (Meenakshi Academy of Higher Education and Research) under specific humidity (65 ± 5%) and temperature (21 ± 2°) with constant 12 h light and 12 h dark schedule. The standard pelleted diet (Lipton India, Mumbai, India) was provided with clean drinking water in *ad libitum.*

Induction of Type-2 Diabetes

Rats were subjected to 60 days of a high-fat diet containing cholesterol 3%, cholic acid 1%, coconut oil 30%, standard rat feed 66%, and 30% sucrose through drinking water. On the 58th day of treatment, after overnight fasting, blood glucose was checked and the rats that had blood glucose >120 mg/dl were chosen as type 2 diabetic rats. Sucrose feeding through drinking water with a high-fat diet was continued until the end of the study.

Experimental Design

Adult male albino rats of Wistar strain 150–180 days old with 180–200 g body weight (b.wt) were randomly divided into four groups of six rats each

Group I : Normal rats

Group II : Type-2 diabetic rats

Group III : Type-2 diabetic rat treated with beta-sitosterol (20 mg/kg b.wt/day), orally for 30 days.

Group IV : Type-2 diabetic rats treated with metformin (50 mg/kg, b.wt/day orally for 30 days).

Two days before killing, control and experimental animals were subjected to oral glucose tolerance test and insulin tolerance test. At the end of the treatment, animals were anesthetized with sodium thiopentone (40 mg/kg b.wt), blood was collected through cardiac puncture, sera were separated and stored at −80°C, and 20 ml of isotonic sodium chloride solution was perfused through the left ventricle to clear blood from the organs. Liver was immediately dissected and used for the further study.

Fasting blood glucose (FBG)

Blood glucose was estimated using On-Call Plus blood glucose test strips (ACON Laboratories Inc., USA) after overnight fasting. Blood was collected by nicking the tip of the rat tail and results are expressed as mg/dl.

Oral Glucose Tolerance (OGT)

For oral glucose tolerance test, animals were fasted overnight and blood glucose was estimated using On-Call Plus blood glucose test strips at various time periods (60, 120 and 180 min) after giving the oral glucose load (10 ml/kg; 50% w/v). Blood glucose value before giving glucose load is considered as 0 minute value. Results are expressed as mg/dl.

Fasting serum insulin

Serum insulin was assayed using ultrasensitive rat insulin ELISA kit obtained from Crystal Chem Inc (Illinois, USA). The range of detection is 0.1 - 64 ng/ml. The percentage cross-reactivity of insulin antibody to rat insulin was 100%. Intra-assay coefficient of variation was ≤10.0% and inter-assay coefficient of variation was ≤10.0%. Results are expressed as ng/ml.

Insulin Tolerance Test (ITT)

This test was performed on random-fed rats. Rats were injected with insulin (0.75 U/kg) in ~ 0.1 ml 0.9% saline intraperitoneally. A drop of blood (5 µl) was taken from the tail vein before the injection of insulin and after 15, 30, 45, and 60 min for the determination of blood glucose with a glucometer. Results are expressed as mg/dl.

Homeostasis Model Assessment for Insulin Resistance (HOMA-IR) and Quantitative Insulin Sensitivity Check Index (QUICKI)

HOMA-IR was calculated using the formula (fasting blood glucose X fasting serum insulin/405) as per the method of Matthews *et al*. (1985) and QUICKI was calculated using the formula 1/(log fasting serum insulin + log fasting blood glucose) as per the method of Katz *et al.* (2000).

Assessment of carbohydrate metabolic enzymes

Standard protocols were used for the assay of glucokinase (Brandstrup *et al*.1957) glucose-6-phosphatase (Hers and van Hoof, 1966), and phosphoenolpyruvate carboxykinase (PEPCK) (Beginsky *et al*., 1992) glycogen synthase (Golden *et al*. 1977) as well as glycogen phosphorylase (Shull *et al*. (1956) by spectrophotometric analysis.

Serum Lipid profile and liver & kidney function markers

Serum cholesterol, triglyceride, LDL, HDL, ALP, AST, ALT, bilirubin, urea, and creatinine were assessed using assay kits purchased from Spinreact, Spain.

Lipid peroxidation and reactive oxygen species

Lipid peroxidation (LPO) was measured by the method of Devasagayam and Tarachand, (1987). The malondialdehyde (MDA) content of the sample is expressed as nmoles of MDA formed/min/mg protein. Hydrogen peroxide generation was assessed by the spectrophotometric method of Pick and Keisari (1981) and expressed as µ moles/min/mg protein. Hydroxyl radical (OH*) production was quantified by the method of Puntarulo and Cederbaum, (1988) and expressed as µmoles/min/mg protein.

Antioxidant enzymes

Superoxide dismutase (SOD) activity was assessed as per the methods of Misra and Fridovich (1972) and results were expressed by units/mg protein. Activity of catalase (CAT)was measured by the method of Takahara *et al*. (1960) and results for the same was expressed as µmoles of H_2O_2 consumed /min/mg protein. Glutathione peroxidase (GPx) activity was measured by spectrophotmetrically as per the standard method (Rotruck *et al,* 1973). GPx level was expressed as µg of glutathione utilized /min/mg protein. Glutathione – S – transferase (GST) was measured by previously published method (Habig *et al,* 1974) and results for the same was expressed as µmol of CDNB utilized /min/mg protein. Glutathione reductase (GH) levels were measured by the mthod of Blakytny and Harding (1992) and expressed as nmoles of GSH/mg protein. Reduced glutathione activity was analyzed (GSH) by the method of Sedlak and Lindsay (1968) and results were expressed as nmoles of GSH/mg protein.

^{14}C-2-deoxyglucose uptake

Glucose uptake was estimated using ^{14}C-2-dexoyglucose as per the method of Ishizuka *et al*. (1999). The results were expressed as CMP of 14C-2-deoxy glucose uptake/10 mg tissue.

^{14}C-glucose oxidation

Glucose oxidation was estimated using ^{14}C-glucose as per the method of Johnson and Turner (1971) & Kraft and Johnson (1972). The results were expressed as CMP of $^{14}CO_2$ released/10 mg tissue.

Glycogen

Glycogen was estimated by the method of Hassid and Abraham (1957). The results were expressed as mg/g wet tissue.

Isolation of protein

Plasma membrane and cytosolic fractions of liver were prepared as described previously (Dombrowski *et al*., 1996; Nevado *et al*., 2006). Insulin receptor substrate-1 (IRS2) and p-IRS2^{Tyr632} proteins were measured in tissue lysate. Insulin receptor (IR)

was measured in plasma membrane whereas as GLUT2 protein was measured in both cytosolic and plasma membrane fractions. The protein concentration was estimated (Lowry *et al*., 1951) using BSA (Bovie Serum Albumin) as a standard.

Western blot analysis of proteins

The lysate proteins (50µg/lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel) and transferred by electroblotting to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, USA). The membranes were blocked with 5% nonfat dry milk and probed with the primary antibodies (which were diluted to 1**:**1000). Following incubation, the blot was washed for three times (5 min each with Tris-buffered saline containing Tween-20 (TBS-T). After washing with TBS-T, membranes were incubated for 1 hour with horseradish peroxidase-conjugated rabbit-anti-mouse or goat-anti-rabbit antibodies (which were diluted to 1**:**5000) (GeNei, Bangalore, India). The specific signals were detected with an enhanced chemiluminescence detection system (Thermo Fisher scientific Inc, USA). The protein bands were captured using Chemidoc and quantified by Quantity One image analysis system, Bio-Rad Laboratories, USA. Later the membranes were incubated in stripping buffer at 50°C for 30 min. After this, the membranes were reprobed using a β-actin antibody (1**:**5000). As the invariant control, the present study used rat β-actin.

Total RNA isolation, cDNA conversion and real-time PCR

Using a TRIR kit (Total RNA Isolation Reagent Invitrogen), total RNA was isolated from control and experimental samples. In brief, to 100 mg fresh tissue, 1 ml of TRIR was added and homogenized. The content was transferred to a microcentrifuge tube instantly and 0.2 ml of chloroform was added, vortexed for 1 min then kept at 4ºC for 5 min. Later, the contents were centrifuged at 12,000 ×g for 15 min at 4ºC.The aqueous phase (upper layer) was carefully transferred to a fresh microfuge tube and an equal volume of isopropanol was added, vortexed for 15 S and placed on ice for 10 min. After centrifugation of the content at 12000 ×g for 10 min at 4ºC, the supernatant was

discarded and RNA pellet was washed with 1 ml of 75% ethanol by the vortex.The isolated RNA was estimated spectrometrically by the method of Fourney *et al.* (1988). The RNA concentration was expressed in microgram (µg).

By using the reverse transcriptase kit from Eurogentec (Seraing, Belgium), complementary DNA (cDNA) was synthesized from 2 µg of total RNA as stated in the manufacturer's protocol. To perform real-time PCR, the reaction mixture containing 2x reaction buffer (Takara SyBr green master mix), Forward and reverse primers of the target gene and house-keeping gene, water and β-actin in total volume of 45 µl expect the cDNA was made, mixed intensively and spun down. In individual PCR vials, about 5 µl of control DNA for positive control, 5 µl of water for negative control and 5 µl of template cDNA for samples were taken and reaction mixture (45 µl) were added. 40 cycles (95ºC for 5 min, 95ºC for 5 s, 60ºC for 20 s and 72ºC for 40 s) was set up for the reaction and obtained results were plotted by the PCR machine (Stratagene MX 3000 P, Agilent Technologies, 530 l, Stevens Creek Blvd, Santa Clara CA, 95051) on a graph. Relative quantification was calculated from the melt and amplification curves analysis.

STATISTICAL ANALYSIS

The data were subjected to statistical analysis using one-way analysis of variance and Duncan's multiple comparison test to assess the significance of individual variations between the control and treatment groups using a computer-based software (SPSS 7.5 using Windows student version). The significance was considered at the level of $p< 0.05$.

RESULT

The result will be discussed later

CONCLUSION

It is concluded from the present findings that, the antidiabetic effect of β-sitosterol in high fat diet and sucrose-induced type-2 diabetic rats is mediated through amelioration of oxidative stress & dyslipidemia, improved insulin signal transduction and GLUT2

expression as well as glucose uptake and oxidation in the liver. Hence, beta sitosterol can be served as one of the therapeutic phytomedicines for the management of type-2 diabetes mellitus.

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